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14. ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor constitutively active in a large number of tumors, where it works as a central player in the activation of multiple oncogenic pathways and contributes to the proliferative state, the migratory and invasive potential and to the maintenance of the mesenchymal phenotype. STAT3 therefore constitutes a potential prime target for directed cancer therapies.

A naturally occurring alternative splicing variant, STAT3β, uses an alternative acceptor site within exon 23 and leads to the production of a truncated isoform, which lacks the C-terminal trans-activation domain (TAD). Depending on context, STAT3β can act as a dominant negative regulator of transcription and promote apoptosis.

We have used modified antisense oligonucleotides to specifically induce a shift of expression from the abundant, active STAT3a to the truncated STAT3β isoform. Induction of the endogenous STAT3β leads to decreased cell viability in cell lines with persistent STAT3 tyrosine phosphorylation, compared to full STAT3 knock-down obtained by Forced Splicing-Dependent Nonsense-Mediated Decay (FSD-NMD). Furthermore, comparison of the molecular effects of splicing redirection to STAT3 knock-down reveals a unique STAT3b transcriptional signature, with the downregulation of specific target genes (including LEDGF, PCAF, Cyclin C, PEX1 and STAT1b) distinct from canonical STAT3 genes typically associated to total STAT3 knock-down.

Here we propose to screen a next generation panel of antisense compounds, to reprogram STAT3 splicing in cancer cells *in vitro* and *in vivo*, in different genetic and orthotopic mouse models, to establish a working model system to test the approach.

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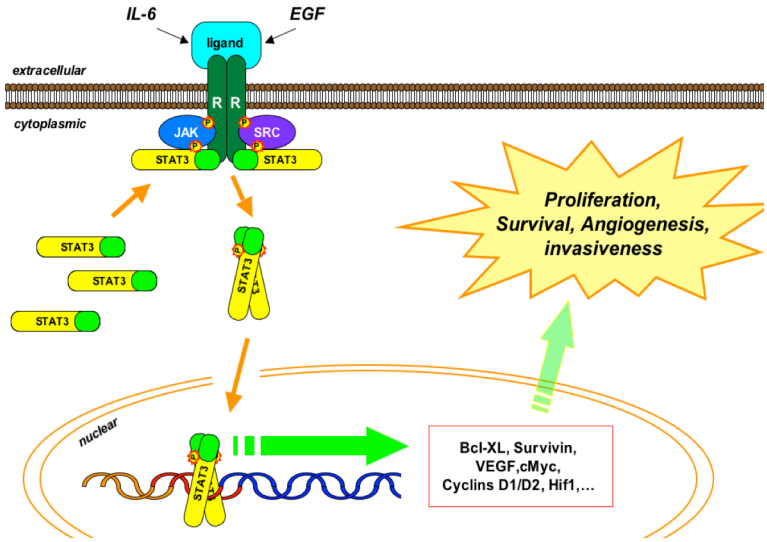
INTRODUCTION

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors implicated in growth factors and cytokines signalling [1]. In the canonical model of STATs signalling, singles monomers are normally sequestered in the cytoplasm in an inactive form. Their activation is initiated by tyrosine phosphorylation, usually mediated by the binding of cytokines or growth factors to their membrane receptors and/or by intracellular oncogenic tyrosine kinases, such as JAKs and Src. Upon cytoplasmic tyrosine phosphorylation, two STAT monomers dimerize, translocate to the nucleus and bind to specific promoter sequences, thereby regulating gene expression[1]. Under physiological conditions, every phospho-STAT protein has a limited activation period that typically lasts from a few minutes to several hours, while persistent activation of STAT proteins, in particular STAT3 and STAT5, is observed in a wide variety of cancers, including breast cancer[2].

STAT3 is implicated in a vast range of physiological processes including cellular proliferation, differentiation, inflammation and immune response[3-6], but can also act as an oncogene to induce cellular transformation and tumorigenesis[7]. Indeed, persistently tyrosine phosphorylated STAT3 has been reported in nearly 70% of haematological and solid tumors[8]. Because of its wide range of functions, STAT3 is involved in many aspects of carcinogenesis such as proliferation, survival, angiogenesis and metastasis[9,10] and can also contribute to tumor escape from immune surveillance[11,12] and to the establishment of tumor drug resistance[13,14]. Consistently, persistent activation of Stat3, has been reported in human breast carcinoma cell lines but not in mammary epithelial cell lines derived from non-malignant tissues[15,16].

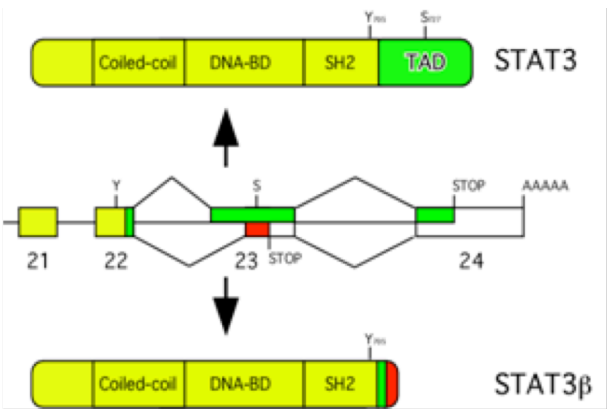
STAT3's pivotal position at the convergence of many oncogenic tyrosin-kinase signalling pathways, makes it a particularly suitable molecular target for cancer therapy,



1. Stat3 signaling. Upon cytokine or growth factor receptor binding, Cyoplasmic Stat3 gets recruited to the plasma membrane and activated via tyrosine phosphorylation by Receptor Tyrosine Kinases (RTKs, such as PDGFR or EGFR), or by non-RTKs (such as Src and JAK). it dimerizes and is translocated to the nucleus where it binds DNA to activate its target genes. Stat3 is tightly regulated by phosphatases, SOCS (suppressor of cytokine signaling) and PIAS (protein inhibitor of activated Stats) and participates into a very complex network of signaling interactions that includes other STAT proteins, additional transcription factors and regulators (not shown).

especially considering that tumor cells tend to become dependent on persistent STAT3 signalling and are more sensitive to its inhibition than normal cells[8,17]. Indeed, direct inhibition of STAT3 activities by multiple means, such as overexpression of dominant negative isoforms[18], antisense oligonucleotides[19], RNAi[20,21] or small drug inhibitors[22] results in growth inhibition and induces apoptosis in breast cancer and other model systems[23]. The direct implication of Stat3 as a promoter of breast tumor growth and progression is further supported by the observation that breast tumor sample show increased Stat3 activity compared to matched non neoplastic tissues, but decreased in samples from patients with a complete pathological response to doxorubicin/docetaxel treatment compared to partial responders[24].

Inihibition of STAT3 activity therefore represents a promising approach in the treatment of breast cancer.

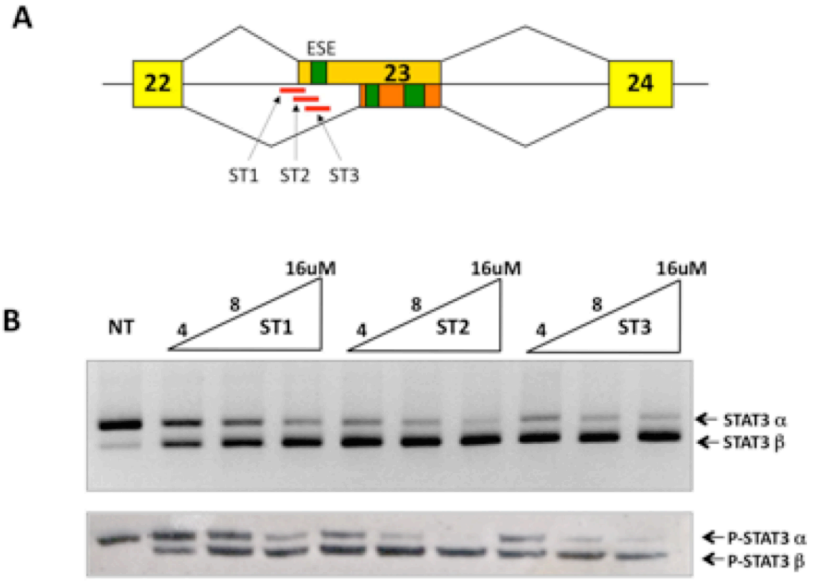


2. STAT3 exon 23 alternative splicing. Alternative acceptor site usage in exon 23 generates both the full length STAT3 transcriptional activator and a dominant negative variant (STAT3 beta) that lacks the transactivation domain (TAD).

A naturally occurring alternative splicing variant, STAT3-beta, uses an alternative acceptor site within exon 23 (Figure 2) and leads to the production of a truncated isoform lacking the C-terminal transactivation domain (TAD). As a consequence, STAT3-beta can still heterodimerize with the full-length STAT3 (STAT3-alpha) and bind to DNA, but it cannot transactivate gene expression, thus effectively blocking STAT3 signalling. Indeed, STAT3-beta can act as a dominant negative regulator of transcription, and its overexpression leads to a significant inhibition of tumor growth and increased apoptosis, both *in vitro* and *in vivo*.

Redirection of STAT3 alternative splicing therefore presents an attractive way to eliminate the tumorigenic STAT3 alpha variant while simultaneously inducing the generation of the STAT3beta variant, which possesses marked anti-tumoral properties.

In the previous CDMRP-BCRP-funded study (BC074961, Modulation of Stat3 Alternative Splicing in Breast Cancer), we induced the STAT3beta isoform using an antisense approach, where modified oligonucleotides were very specifically targeted to splicing regulatory elements to either inhibit or promote the usage of splicing sites[25]. Use of



3. Modulation of Stat3 splicing(A) Schematic localization of the morpholino oligonucleotides relative to exon 23 a 3' splice site and putative mapped ESEs (shown in green). **(B**) (Top) RT-PCR and (Bottom) Western Blot analysis of STAT3 a/b levels in MDA-MB-435s cells treated with increasing concentration of ST1, ST2 and ST3 morpholino oligonucleotides for 4 days.

antisense compounds is a well-established approach for gene expression regulation[26], and normally relies on the destabilization of the target mRNAs, either by triggering RNase H degradation or by taking advantage of the RNAi mechanism. Redirection of splicing differs from the standard antisense-based approach in that the objective is not degradation of the target RNA. On the contrary, triggering of RNase H must be avoided in order to preserve the integrity of the re-directed mRNA, and therefore different chemical characteristics must be present in the antisense compounds.

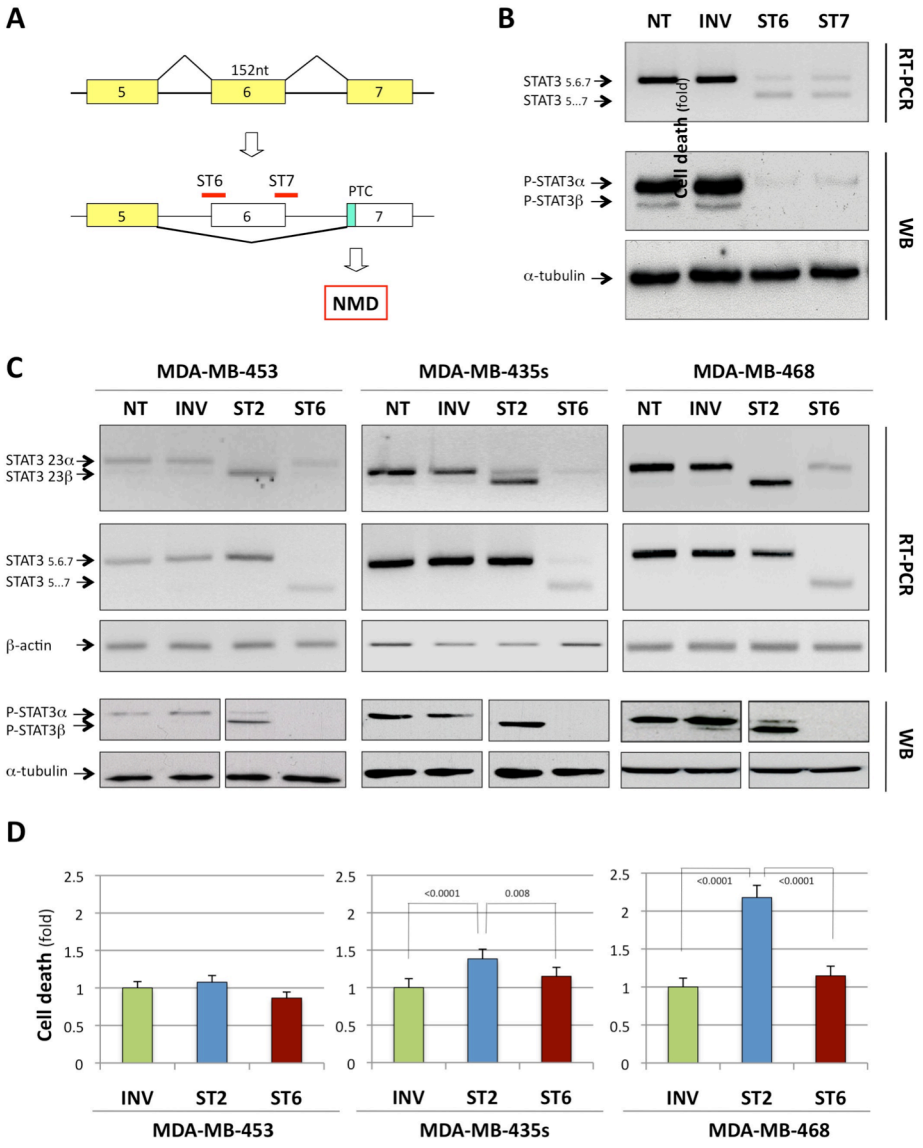
Morpholino phosphorodiamidate oligonucleotides (morpholinos) are DNA oligonucleotide analogues in which the phosphodiester bond is replaced by phosphorodiamidate linkage and the ribose is replaced by a morpholino moiety. Because of its noncharged backbone, morpholinos form very stable duplexes with single-stranded RNA targets and are highly resistant to nucleases and proteases. We had previously developed chimeric compounds that are coupling to the antisense moiety of a peptide (RS-rich) that mimics the action of the "RS domain", the 'activation domain' of splicing factors[27]. In addition, Arginine-rich peptides share structural similarities to translocating peptides (like TAT) that are able to mediate free-uptake of bound cargo into cells. In addition, we have also used a formulation of the compounds (vivo-morpholino) where a cationic dendrimer is coupled to the oligonucleotide instead of the peptide. This variant was originally developed by Gene-tool for *in vivo* treatments.

We thus used splicing redirection compounds to induce a switch from the alpha to the STAT3beta isoform (Figure 3).

To be able to compare the effect of the beta switch to those of a STAT3 knock-down, we adapted the splicing redirection approach to cause a total knock-down by inducing an early splicing shift of a constitutive exon, which leads to a frameshift, a Premature Termination

Codon (PTC) and eventually to degradation of the mRNA by nonsense-mediated decay (NMD). The resulting fsd-NMD (forced splice-dependent NMD) was used to completely knock-down Stat3 as a control (Figure 4 a,b). We then started to assess the biological effect of the switch in breast cancer cell lines that have different extent of Stat3 dependence. The activity was measured using a number of cell-based biological assays.

The alpha-to-beta switch induces cell death in triple-negative Stat3-dependent cell lines like MDA-MB-435s or MDA-MB-468, but not in cell lines such as MDA-453, that do not show persistent activation of Stat3 (figure 4c,d). In side-by-side experiments, however, full knock-down of Stat3 was not as effective as the splicing switch in promoting cell death, suggesting that Stat3beta may induce cell death



4. Effect of STAT3 alternative splicing modulation *in vitro* (**A**) Schematic representation of the strategy used to knock-down STAT3 by Forced Splice-dependent Nonsense-Mediated Decay (FSD-NMD). Morpholino oligonucleotides ST6 and ST7 cause skipping of exon 6 leading to a frameshift and a premature termination codon (PTC), ultimately causing RNA degradation following nonsense mediated decay (NMD).
(**B**) RT-PCR and Western Blot analysis of STAT3 α/β levels in MDA-MB-435s cells treated with 16μM of ST6, ST7 or INV for 4 days. α-tubulin was used as loading control.
(**C**) RT-PCR and Western Blot analysis of STAT3 α/β levels in MDA-MB-453, MDA-MB-435s and MDA-MB-468 cells treated with 16μM of INV, ST2 or ST6 oligos for 4 days. β-actin was used as housekeeping gene for the RT-PCR and α-tubulin was used as loading control for Western blot. NT, untreated.
(**D**) Cell death quantification (represented as fold change relative to control treatment) determined by trypan blue exclusion for MDA-MB-453, MDA-MB-435s and MDA-MB-468 cells treated with 16μM of INV (green), ST2 (blue) or ST6 (red) oligonucleotides for 4 days. Data represent average of ≥ three independent experiments (each experiment done in triplicates). Indicated p-values were calculated by the stratified chi-square test, bars represent standard error of the rate (SE).

by a mechanism different than a straight dominant negative effect.

Indeed, we found that the alpha-to-beta switch activates a specific transcription program that includes downregulation of survival factors, including LEDGF, PCAF, Cyclin C and Stat1beta. Next, we tested whether the splicing redirection compounds also possess anti-tumor activity *in vivo*. The beta-switching compound ST2 and the knock-down compound ST6 were injected intratumorally in athymic mice carrying 435s-derived xenograft tumors [25]. The switch to beta was associated to a full regression of the tumors providing the first evidence that modulation of a single splicing event can have anti-tumoral properties. On the other hand, knock-down of total STAT3 didn't have a significant effect on tumor growth even if the treatment was very effective at the molecular level [25].

BODY

Role of Stat3beta downstream effectors [Cartegni Lab]

Our previous experiments showed that reduction of STAT3a and concomitant induction of STAT3b by alternative splicing modulation can decrease cancer cell viability more efficiently than the knockdown of both STAT3 isoforms. Therefore we set out to determine which STAT3 target genes may be involved in this process.

We thus performed a screen using cDNAs from MDA-MB-435s cells treated for 4 days with ST2 (STAT3b induction), ST6 (STAT3 K.O.) or INV (control) compounds. Expression levels were analyzed by real-time PCR, using the microarray platform U133A 2.0 (Affymetrix) [25]. Whereas knocking-down both STAT3 isoforms by FSD-NMD using the ST6 compound resulted in a somewhat modest but consistent downregulation of most canonical targets analyzed (and a robust downregulation of STAT3 itself, as expected), STAT3b induction by ST2 appears to have activated a STAT3b-specific transcriptional signature [25], indicative of the existence of different classes of target genes in terms of how they respond to STAT3a/STAT3b regulation.

Out of the panel of genes with statistically significant variation of expression levels in the microarray assay, in addition to the previously described STAT3-target interleukin 8 (IL8), five other genes were confirmed to be down-regulated both at the RNA and protein level by the switch to STAT3β, but not by the knockdown: 1.) **Lens Epithelium-Derived Growth Factor** (LEDGF/PSIP1, a chromatin binding protein and transcriptional co-activator; a pro-survival and growth factor)[28]; 2.) **peroxisomal biogenesis factor 1** (PEX1, part of the AAA ATPase

subfamily, required for peroxisomal import)[29]; 3.) **CyclinC** (CCNC, controls transcription by associating with Cdk8 and modulating RNA pol II activity and regulates G0/G1 transition) [30]; 4.) **p300/CBP-associated factor** (PCAF, histone acetyltransferase and transcriptional co-activator, promotes growth, invasion and drug resistance)[31]; and 5.) **STAT1** (proliferative antagonist isoform of tumor suppressor STAT1)[32]. In the case of STAT1, the effect is indirect or post-transcriptional, as only the alternative splicing variant STAT1b, but not the full-length variant STAT1a is affected). Notably, the microarray analysis confirmed that none of the canonical STAT3 targets, except for IL8, were strongly down-regulated by the STAT3β induction. LEDGF, PEX1, Cyclin C and STAT1b were also specifically down-regulated by the switch from STAT3α to STAT3β in MDA-MB-468 cells.

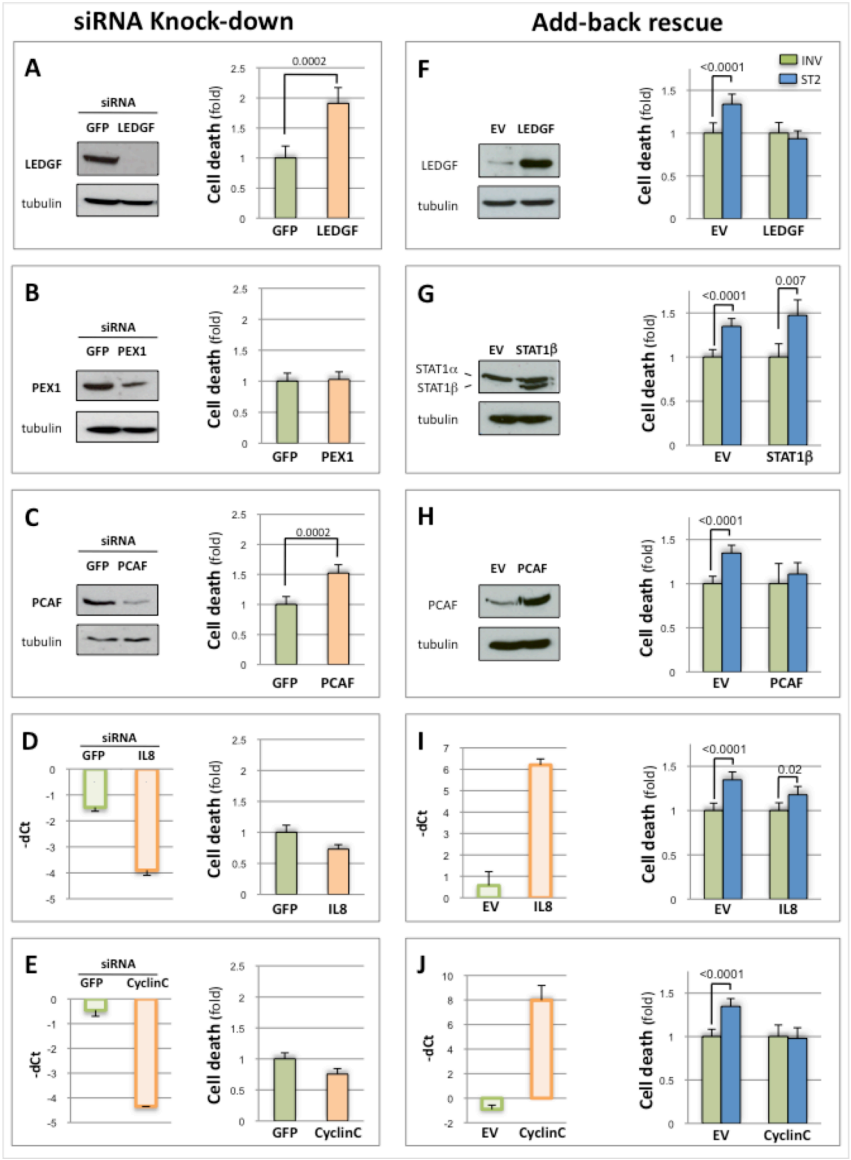


Figure 5. Knock-down and overexpression of STAT3β target genes. (**A-E**) (Left) MDA-MB-435s were treated for 72 hours with siRNA against GFP or: LEDGF (A), PEX1 (B), PCAF (C), IL8 (D) or CyclinC (E). Effective knock-down was verified by WB using specific antibodies (A-C, with α-Tubulin as control) or by qPCR analysis (D-E), displayed as -dC(t) values after normalization to HPRT. (Right) Cell death quantification determined by trypan blue exclusion for MDA-MB-435s treated as described. Data are displayed as fold change of treated samples (orange) relative to control treatment (green) and represent average of three independent experiments (each in triplicates). P-values were calculated by the stratified chi-square test, bars represent standard error of the rate (SE). **(F)** (Left) Lysates from MDA-MB-435s transiently over-expressing LEDGF or transfected with empty vector (EV) were immunoblotted using antibody to LEDGF. (Right) The transfected cells were concurrently treated with 16uM of INV or ST2 morpholinos for 4 days, and cell death was quantified as above. **(G-J)** (Left) Stable MDA-MB-435s clones over-expressing STAT1β (G), PCAF (H), IL8 (I), CyclinC (J) or selected for the empty vector (EV) were treated with 16uM of INV or ST2 morpholinos for 4 days. Over-expression levels were determined by WB using specific antibodies (G-H, with α-tubulin used as loading control) or by qPCR analysis (I-J), displayed as -dC(t) values after normalization to HPRT. (Right) Cell death quantified as above.

Knockdown and rescue of STAT3β targets [Cartegni Lab]. The set of target genes specifically down-regulated when STAT3 splicing is redirected from STAT3α to STAT3β had not previously been associated with STAT3β activity. In order to get a better understanding

of their contribution to the biological effects observed, we took two different approaches. First, we tested whether their individual down-regulation was able to recapitulate the decrease in cell viability observed with the ST2 treatment. MDA-MD-435s cells were treated with siRNA against five target genes (no siRNAs are available to specifically target STAT1b but not STAT1a). Effective knock-down was verified by qPCR and WB, where suitable antibodies were available (Figure 5 A-E) and cell viability was measured. Individual down-regulation of PEX1, IL8 or CyclinC did not lead to an increase in cell death (Figure 5B, D, E). On the other hand, knock-down of LEDGF (Figure 5A) and PCAF (Figure 5C), was associated to a significant decrease in cell viability.

Next we asked whether forced re-expression of the STAT3β target genes could protect from the increase in cell death induced by the α-to-β switch. We thus generated MDA-MB-435s-derived cell lines stably expressing four of the STAT3β target genes (STAT1b, PCAF, IL8, CyclinC). Initially we were unable to generate a stable LEDGF-expressing line, therefore the equivalent experiment (shown) was performed under transient expression conditions. However, additional stable clones are under characterization and once obtained will be also used for successive phases of the proposed experiments.

Cells were concurrently treated in vitro with either ST2 or INV compounds, and cell viability was measured after 4 days. Forced expression of STAT1b did not protect from cell death induced by the splicing switch (Fig. 5G), compared to cells selected for integration of the empty vector. Expression of IL8 (Fig. 5I) showed a reduced but still significant increase in cell death.

Most importantly**, LEDGF, PCAF and Cyclin C** protected cells from the α-to-β shift (Fig. 5F, 5H-J) as treating the overexpressing cells with ST2 had no effect on their viability, unlike what observed with control cells. Taken together these data suggest that the effect of the STAT3β isoform on cell viability is likely mediated by the combined down-regulation of a specific set of target genes rather than exerted through a single major effector, in agreement with STAT3 pleiotropic functions in the pathogenesis of cancer.

In vivo treatment of stat3-dependent tumors [Cartegni lab, de Stanchina lab]

In preliminary experiments, we have tested how STAT3 alternative splicing modulation could affect tumor growth in vivo, using the ST2, ST6 and INV morpholino compounds described earlier, delivered by intratumoral injection. Athymic mice subcutaneously implanted with MDA-MB-435s cells were IT injected twice a week for a total of 3 weeks while monitoring for tumor growth. 13 days after the first injections tumors treated with the switching ST2

morpholino showed regression (Figure 5A-B) while tumors treated with the knock-down ST6 compound grew as well as PBS and INV-treated tumors [25].

RT-PCR for either STAT3 exon 23 (Figure 5C) or STAT3 exon 6 (Figure 5D) confirmed efficacy of both ST2 and ST6 treatments in inducing either STAT3 splicing switch or total STAT3 FSD-NMD knockdown. Tumor sections were analyzed by immunohistochemistry (IHC) with three STAT3 antibodies. Staining for total-STAT3 and P-Y705 (present in both STAT3α and STAT3β) was strongly decreased in ST6-treated tumors (Figure 5F, 5H), confirming that the ST6 compound was effective at substantially knocking down STAT3. On the contrary, the level of both total-STAT3 and P-Y705 in ST2-treated tumors was comparable to control-treated tumors, consistent with the persistent expression of STAT3β and the maintenance of this phosphorylation site (Figure 5F, H). IHC for P-S727 (present only in STAT3α) was negative for both ST2 and ST6 treated tumors, further confirming the efficacy of these treatments at the protein level (Figure 3G). Analysis of tumor sections stained for Hematoxylin and Eosin (H&E, Figure 3I), showed that the ST2-induced splicing switch is the only treatment associated with a significant effect on tumor morphology. Of particular interest is that while STAT3 knock-down was effective at the

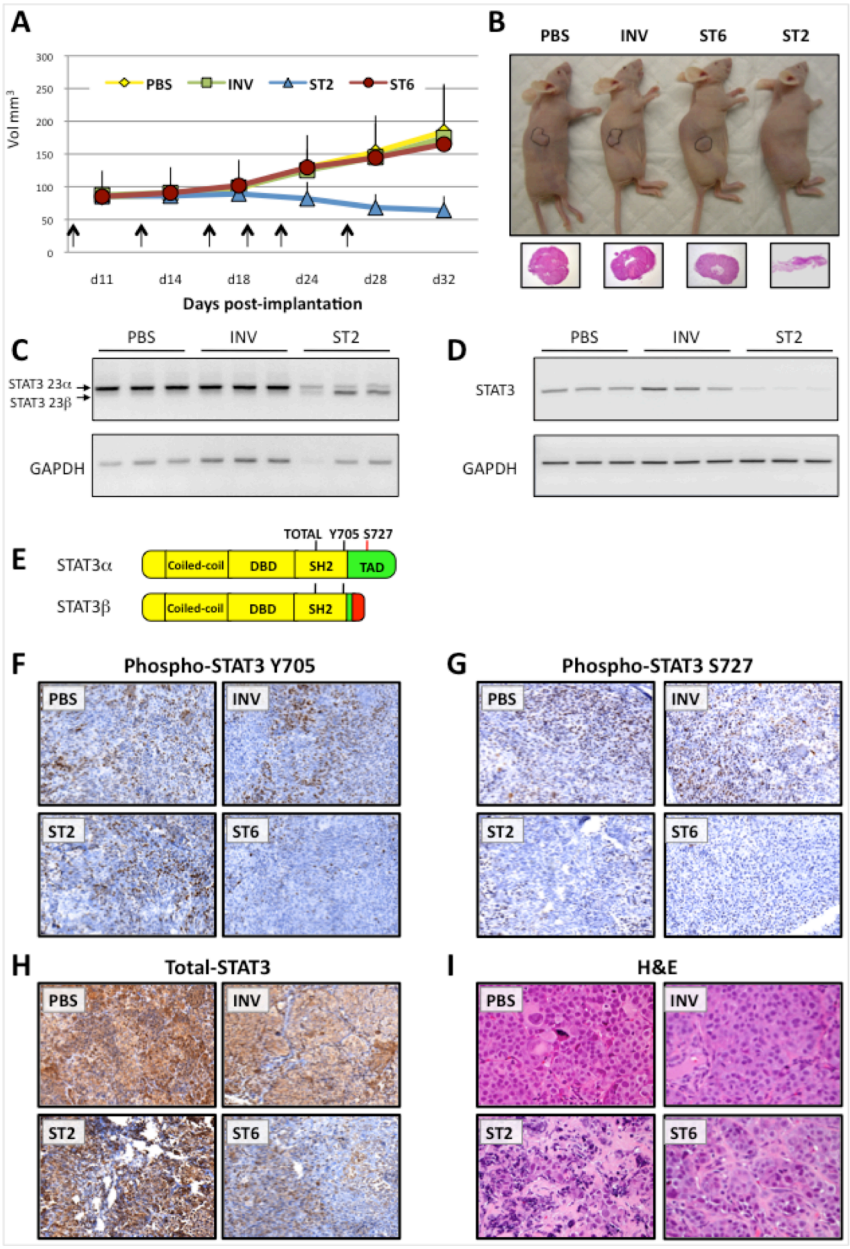


Figure 5. Effect of STAT3 alternative splicing modulation *in vivo.* (**A**) Following subcutaneous injection of $10x10^6$ MDA-MB-435s cells, tumors of ~100 mm^3 were treated intratumorally with 0.12mg of vivo-Morpholino (PBS, INV, ST2 and ST6) twice a week for 3 weeks (arrows) and tumor volumes were measured. **B**) Representative image of mice in each treatment group at time of sacrifice, with H&E staining of tumor sections underneath. (**C, D**) RT-PCR analysis of STAT3 exon 23 and exon 6 levels (respectively) in three tumors for each treatment group. GAPDH was used as control. **(E)** Epitopes' location of STAT3 antibodies used for the IHC staining. **(F, G and H)** Representative images of tumor sections from the 4 different treatment groups following IHC using specific antibodies against P-Y705, P-S727 and total STAT3, respectively. **(I)** Representative images of H&E stained tumor sections from the 4 different treatment groups.

molecular level, it did not have any notable effects on tumor morphology and growth compared to controls (Figure 3I).

Expression of LEDGF, Cyclin C, STAT1b and PCAF and IL8 is also inhibited in MDA-MB-435s-derived tumors in mice upon STAT3b activation, like previously described for the MDA-MB-435s cell lines. To assess whether their suppressive role in STAT3b-induced cell death also applies to in vivo tumor growth and progression when overexpressed, we started set up an in vivo system using the stable cell lines described above.

In experiments carried out in the de Stanchina lab, new baselines for tumor growth were derived in 8-10 weeks old athymic female mice, which were subcutaneously inoculated with 10X106 MDA-MB-435s cells expressing STAT1b and PCAF. In addition, MDA-MB-435s-derived CyclinC and LEDGF cell lines are being currently re-generated from novel individual clones, as the expression in the previous clones resulted unstable. Next, a set of experiments using MDA-MB-435s cells overexpressing PCAF, STAT1b and control MDA-MB-435s, divided in 3 treatment group each (mock, INV and ST2 for splicing re-direction, 5 mice per group) have been started, with Vivo-Morpholino administered intratumorally at a final concentration of 0.12mg/30ul, twice a week. Tumor-bearing mice are assessed for weight loss and tumor volume twice weekly. Tumor volumes were determined by caliper. Tumor samples were harvested, to be analyzed by immunohistochemistry and at the RNA and protein levels. Unfortunately there was an issue with tumor up-take, so the results of these experiments are not conclusive and will need to be repeated for statistical significance. However, our initial indications suggest that in the presence of overexpressed PCAF (and to a lesser extent STA1b) the treatment with the splice-switching ST2 vivo-morpholino compound leads to a reduction in tumor growth, although such reduction does not appear to be as effective as when PCAF is not over-expressed, in which case a full tumor regression is observed. This is important, because (if confirmed) it suggests that also in this xenograft in vivo system, PCAF plays a key role in mediating Stat3beta anti-tumoral activity.

As well as repeating and completing the experiment delineated above, we have also designed FSD-NMD morpholino compounds to knock-down endogenous PCAF expression, and to induce endogenous expression to activate STAT1b. These have now been synthesized to be tested for activity in cell lines [Cartegni lab], Active compounds will then be used by themselves and in combination to test their anti-tumoral activity, to show that the anti-tumorigenic activity of the ST2 compounds is mediated by PCAF and/or STAT1b [de Stanchina lab].

In parallel, we have designed and synthesized morpholino compounds specific to either the human (ST2h) or mouse STAT3 exon 23 sequence (ST2m), which induce activation of

STAT3b in either species but not in both. These have now been cross-tested in human and mouse cell lines to confirm that they can effectively and specifically activate STAT3beta in a species-specific way [Cartegni lab]. Similarly to previous experiments, the comparison in vivo using the MDA-Mb-435s model to compare targeting the tumor proper exclusively (ST2h) vs. targeting the tumor environment exclusively (ST2m) vs. targeting both (ST2) are ready to be undertaken in the de Stanchina lab.

Finally, next-generation compounds, based on alternative chemistry (2'MOE) and alternative delivery system (peptide-based) have been designed and synthesized and initial testing are undergoing in the Cartegni lab. Similarly, initial optimization procedures to establish a second, complementary model of aggressive breast cancer using MDA-MB-231 subpopulation LM2-4175[33] cells subcutaneously (as above), systemically (via injection into the left ventricle) or orthotopically into axillary mammary fat pads of 6-week old female athymic nude mice are underway in the de Stanchina lab. This cell line expresses luciferase, which allows for accurate quantification of tumor growth, and induces lung metastases, which will allow us to test for compound efficacy in metastatic conditions. For mammary-fat-pad tumor assays, 3 million cells resuspended in a 50:50 solution of PBS and Matrigel are directly injected into the mammary fat pad corresponding to gland #3. For cardiac injections, 1 million cells resuspended in 100 ul PBS are injected into the left ventricle of anesthesized mice. Tumor progression is visualized by weekly imaging. To do so, mice will be injected with D-luciferin (Xenogen) at 50 mg kg and Photonic emission captured with the In Vivo Imaging System (IVIS, Xenogen). Tumor bioluminescence is then quantified by integrating the photonic flux (photons per second) through a region encircling each tumor, as determined by the LIVING IMAGES software package (Xenogen).

Calibration experiments for imaging have been performed and usage of this model with the ST2 compound will begin in the next funding period.

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KEY RESEARCH ACCOMPLISHMENTS

1. **Generation of improved stable MDA-MB-435s cell lines overexpressing LEDGF and CyclinC**
2. **Effect on tumor growth of overexpression of PCAF or STAT1b**
3. **Effect of STAT3 alpha-beta induction on growth of tumors overexpressing PCAF or STAT1b**
4. **Design and testing of morpholino compounds targeted to PCAF (FSD-NMD) and STAT1b (splice-switching)**
5. **Design and testing of morpholino compounds specifically targeted to mouse or human STAT3 exon 23**
6. **Design an testing of 2'MOE compounds**
7. **Establishment and calibration of the MDA-MB-231 (LM2-4175) model system**

REPORTABLE OUTCOMES

1. **Lee Spraggon and Luca Cartegni;** Antisense Modulation of RNA Processing as a Therapeutic Approach in Cancer Therapy, *Drug Discovery Today: Therapeutic Strategies* (2013) http://dx.doi.org/10.1016/j.ddstr.2013.06.002

CONCLUSIONS

STAT3 is an oncogene transcription factor whose constitutive activation in a large majority of tumors, including breast cancer, is thought to contribute to multiple aspects of the tumorigenesis process. Its main alternative splicing isoform, STAT3b, can act as a dominant negative factor that, when overexpressed in cancer cells, is able to inhibit cell growth in vitro and in vivo. In addition to its dominant negative functions. STAT3b is also in direct transcriptional control of some specific pathways, with antitumorigenic properties, which are independent of the full length STAT3 activities. This Sta3b-specific targets include PCAF, LEDGF, IL8, Cyclin C and STAT1b.

We have previously shown that the modulation of STAT3/STAT3b relative expression levels via splicing re-direction compounds, can lead to a potent anti-tumorigenic approach trough the modulation of the above mentioned STAT3b specific set of target genes.

The first funding period of this project has been instrumental in the setup of numerous experimental systems, with the multiple targets described in the specific aims of the project. In particular, a lot of the early work in the Cartegni lab has been focused on the exploration of the specific role of the a few STAT3b targets (PCAF, LEDGF, IL8, STAT1b) with the determination of their causal involvement in anti-tumorigenic activities.

In addition, the design, synthesis and initial testing of new compounds to regulate the above mentioned targets and to develop next generation, more active STAT3 splicing redirection compounds, or compounds that interrogate the role of STAT3beta in the tumor proper or in the tumor environment has been carried out in the Cartegni lab.

At the same time the de Stanchina lab has proceeded in the establishment of multiple novel in vivo systems, including those aimed at assessing the role of the above mentioned STAt3b targets in vivo, those aimed at testing the role of Stat3beta in the tumor microenvironment and those directed at the establishment of a second, aggressive breast cancer system which incorporates a luciferase readout, This will also provide the possibility to assess the effectiveness of all the above compounds (first and second generation) by themselves or in combination with traditional chemotherapy in a metastatic model of breast cancer.

A major hurdle, which has been associated with the interruption of the research project at the end of the first funding period, has been the transfer of the laboratory of Dr. Cartegni's from Memorial Sloan-Kettering Cancer Center, New York, to the Susan Lehman Cullman

Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers University, New Jersey.

The transfer initiated at the end of September 2013 has taken significantly more time than expected and the new laboratory has became fully operative only in February 2014. In addition to the 4 months ‘down-time’, additional delays compared to the original timeline are expected because of the need to train and bring up to speed the new personnel tha have been hire for the task.

Importantly, no DOD funds from this grant have been used, at MSK or at Rutgers, since the move has started in September 2013 in order to wait for the laboratory to become fully operative and the fund situation to be clarified. No changes occurred within the lab of Dr. de Stanchina (MSKCC), so nothing is expected to change regarding that part of the project, although experiments have been suspended for the delay period in her laboratory as well.

Lehman Cullman Laboratory for Cancer Research at Rutgers university is a prestigious institution that will fully provide all the needed equipment, support and expertize needed to carry out the project as anticipated.

The only significant difference anticipated is that we will be asking for a no-cost six months extension at the end of the final funding period to compensate the delay due to the move of Dr. Cartegni’s lab.

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APPENDICES

SUPPORTING DATA

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Antisense Modulation of RNA Processing as a Therapeutic Approach in Cancer Therapy

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Abstract

Next-generation antisense technologies are re-emerging as viable and powerful approaches to the treatment of several genetic diseases. Similar strategies are also being applied to cancer therapy. Re-programming of the expression of endogenous oncogenic products to replace them with functional antagonists, by interfering with alternative splicing or polyadenylation, provides a promising novel approach to address acquired drug resistance and previously undruggable targets.

Introduction

Cancer develops when a series of genetic changes allows a cell to break free from the normal regulatory pathways governing cell division and proliferation. Its progression is characterized by the appearance of common steps, which include sustained proliferative signaling, evasion of growth suppressors, resistance to apoptosis, activation of replicative immortality, angiogenesis, evasion from host immune response, reprogramming of metabolic pathways, genomic instability, tumor-promoting inflammation, invasion and metastasis [1,2]. These ”hallmarks of cancer” are typically driven by genetic alterations that involve gain-of-function mutations and/or over-expression of oncogenes on one hand, and loss-of-function mutations and/or silencing of tumor suppressors on the other.

Cancer therapy has traditionally been based upon the combination of chemotherapy and radiation. In recent years, the molecular characterization of cancer through genomic, transcriptome and proteomic technologies has led to the development of “targeted therapies” that specifically hit pathways essential for cancer growth and progression [3,4]. These treatments, usually in the form of small molecule inhibitors or antibodies, have revolutionized cancer therapy and dramatically impacted survival rates [5]. However, because of the consistent emergence of resistance to conventional and targeted therapies in tumor cells, and due to the “undruggability” of many promising putative therapeutic targets, there is a persisting requirement for novel alternative drug discovery strategies.

Recent advances in nucleic acid antisense-based technology, comprising improved chemistry, pharmacology and delivery, have led to the development of a new generation of antisense oligonucleotide compounds (ASOs) with greatly improved pharmacokinetic and pharmacodynamic properties [6]. ASOs are emerging as a powerful class of drugs that can be generated against virtually any RNA --coding or non-coding-- and their potential thus surpasses traditional drug discovery based on small molecule inhibitors or monoclonal antibodies. These features have led to the FDA approval of the first antisense-based drug in two decades (*Mipomersen* [7]), with many more currently in clinical trial for the treatment of numerous diseases [8,9], including several for cancer (Table 1).

“Antisense technology” is a broad umbrella term to indicate any approach that involves base-pairing of a compound to a target nucleic acid. It can involve diverse mechanisms of action, ranging from multiple ways to promote target RNA degradation (e.g. by RNA interference (RNAi)), to translation interference, to modulation of non-coding RNA activities and to various pre-mRNA reprogramming strategies (Box 1). In this review, we will focus on the use of synthetic ASOs to force specific changes in endogenous alternative splicing (AS) and alternative polyadenylation (APA) events and their application as a therapeutic avenue for cancer therapy.

Antisense oligonucleotides: Knockdown vs. reprogramming

In the discussion of antisense-based compounds, it is important to distinguish whether the primary goal of the treatment is to downregulate the target gene or to alter its coding potential.

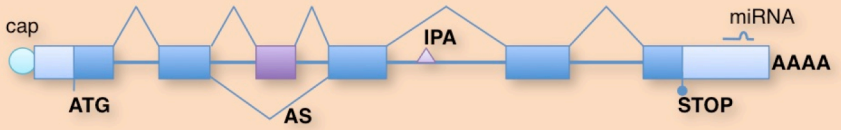
Target knockdown can be achieved by various mechanisms. The most commonly used one exploits the RNAi pathway, which is activated by small

Table 1 – Antisense Oligonucleotides in Clinical Trials for Cancer

Name	Target	ASO	Phase	Cancer Types
Custirsen	Clusterin	2’-MOE	III	Prostate and NSCLC
ISIS EIF-4ERX	EIF-4E	2’-MOE	II	Prostate and NSCLC
OGX-427	HSP-27	2’-MOE	II	Bladder and NSCLC
ISIS-STAT3RX	STAT3	2’-MOE	I/II	Advanced Tumors
AEG3516	XIAP	2’-MOE	I/II	Advanced Tumors
Oblimersen	BCL-2	PS	I/II/III	Lymphoma, AML, CLL, SCLC, RCC
AP12009	TGFb2	PS	I/IIb/III	Glioblastoma
Cenersen	P53	PS	II	CLL

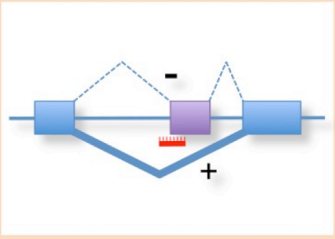
2’MOE= 2’-O-methoxyethyl , PS=Phosphorothioate

Box I. Antisense-based strategies



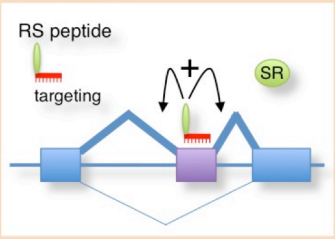
The expression of any typical multi-exon gene, represented above, can be controlled by antisense compounds by a multiplicity of mechanisms. Some target pre-mRNA processing in the nucleus, like splicing or polyadenylation, affecting both levels and structure of the protein products. Others lead to mRNA degradation by various nucleases or can modulate translation, both negatively and positively.

a. Alternative splicing re-direction



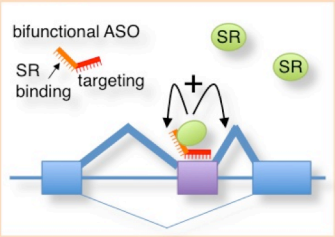
RNAse H-independent ASOs targeted to splice sites or regulatory elements prevent snRNPs or RNA-binding proteins from recognizing them. Splicing is thus redirected to the alternative variant. The effect is typically inhibition of the selected event, but it can be enhancement if a silencer is targeted.

b. ESSENCE



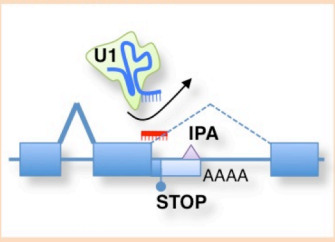
Exon-Specific Silencing ENhancement by small Chimeric Effectors (ESSENCE). ASOs are coupled to an RS peptide to mimic the 'splicing activation domain' of an SR protein. The RS peptide recruits spliceosomal components by protein:protein interactions to acti-vate splicing of nearby splice sites (described in Cartegni *et al.*, [88]).

c. TOES



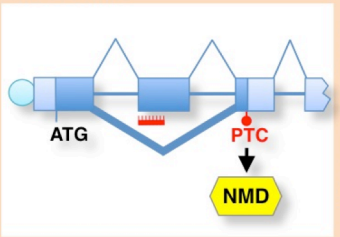
Targeted Oligonucleotide Enhancers of Splicing (TOES). Bifunctional ASOs are used, that include a targeting moiety and a high-affinity binding site for splicing factors (e.g. SR proteins). SR proteins are recruited by the ASOs and thus activate splicing (described in Owen *et al.*, [89]).

d. IPA Activation



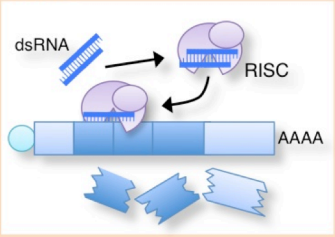
U1snRNP normally inhibits use of downstream intronic polyadenylation sites from its position at the 5'ss. Targeting ASOs to the 5'ss prevent U1 binding and releases IPA, leading to a truncated polyadenylated mRNA (described in Vorlova *et al.*, [79]).

e. FSD-NMD



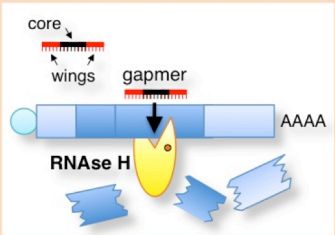
Forced Splice-Dependent Nonsense Mediated Decay (FSD-NMD). ASOs targeted to an early out-of-frame exon induce exon skipping, causing a frameshift. The resulting PTC triggers NMD and thus mRNA degradation (described in Zammarchi *et al.*, [73]).

f. RNAi



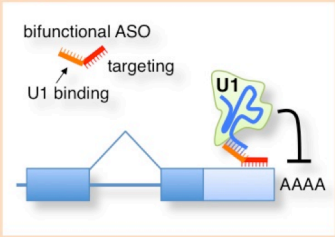
Short double stranded RNAs (siRNAs or shRNAs) are processed and incorporated into the RNA-induced silencing complex (RISC) as templates to recognize target mRNAs, which are then degraded by the nuclease activity.

g. RNAse H-dependent degradation



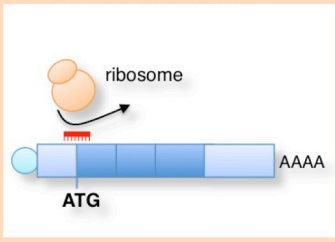
DNA:RNA heteroduplexes are recognized by RNAse H and the RNA is degraded. RNAse H-dependent ASOs recruit RNAse H to the target mRNAs and direct their degradation. Modern compounds use chimeric gapmers, with a RNAse H-triggering core surrounded by protective wings.

h. U1i



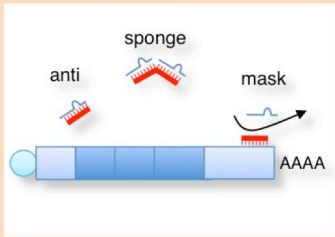
U1 interference (U1i) U1 snRNP can suppress polyadenylation when bound upstream of a PAS. Bifunctional U1 adaptors can be used to tether U1 snRNP upstream of any given PAS, blocking polyadenylation and thus silencing the gene (described in Goraczniak *et al.*, [82]).

i. Translation interference



Morpholinos or other RNaseH-independent ASOs are directed to the ATG region of an mRNA to prevent its recognition by the ribosome, and thus inhibit translation (described in Summerton, [90]).

j. miRNA block



MicroRNAs bind to sites on mRNAs and silence genes by repressing translation. They can be blocked by complementary ASOs or RNAs that directly bind them (anti-miRNAs or miRNA sponges), or that compete for their binding sites (miRNA masks). The treatment results in activation of protein expression (described in Ebert *et al.*, [91]).

interfering RNAs (siRNAs), or short hairpin RNA (shRNAs). These double-stranded RNAs are loaded into the RNA-Induced Silencing Complex (RISC) and repress expression by base-pairing with the target mRNAs and directing their cleavage and degradation [10]. SiRNAs and shRNAs have been utilized *in vivo* with some success, and several RNAi-based compounds are in clinical development [9,11]. However,

their current therapeutic appeal is still somewhat hindered by the requirement for complex formulations and unsatisfactory delivery *in vivo,* particularly to tumors.

While the lion's share of antisense applications (especially in vitro) is currently held by RNAi technologies, the use of ASOs to inhibit gene expression was pioneered in 1978 by Zamecnik and Stephenson --long before the discovery of RNAi-- when ASOs complementary to a Rous sarcoma virus RNA target sequence were shown to repress viral replication [12]. Since then, multiple classes of ASOs have been developed, and while they share the defining targeting by complementary Watson-Crick base-pairing and an average length of ~20 nucleotides, they involve very different chemistries which exploit multiple underlying biological mechanisms [13] (Box 1).

"Classical" ASOs downregulate target RNA by recruiting ribonuclease H to the ASO:RNA duplex, resulting in RNAseH-mediated cleavage of the RNA moiety. They typically carry phosphorothioate (PS) backbones, the most widely used chemistry of the initial antisense technologies, and the basis for *Fomivirsen*, the first and -until the approval of *Mipomersen*- the only FDA-approved ASO drug [14]. Additional modifications coupled and/or alternative to PS have resulted in second and third generation compounds with reduced toxicity, enhanced stability *in vivo*, better tissue distribution and higher targeting efficiency [6,13]. These modifications include substitutions of the ribose groups with 2'-O-Alkyl groups (2'-O-methyl [2'-OME] or 2'-O-methoxyethyl [2'-MOE]), and 2'Fluoro (2'F) or Lock Nucleic Acid (LNA) modifications, which can reduce or prevent the recruitment of RNAse H. Therefore, when the desirable outcome is the knockdown of the target gene, they are typically used in a chimeric "gapmer" context as protective wings at the sides of a core region utilizing simple DNA or other RNAseH-activating chemistries [6,13] (Box1). Other common non RNAseH-activating modifications include replacing the sugar backbone with morpholino rings (phosphorodiamidate morpholino [PMO] oligonucleotides), or with peptoid-like chemistry (peptide nucleic acid [PNA] oligonucleotides) (reviewed in Kole et al., [6]).

Importantly, the same modifications that do not trigger RNA degradation also allow for their usage in applications where the primary goal is not knockdown, but rather the reprogramming of target pre-mRNAs to express alternative, more desirable variants by AS or APA.

Alternative splicing and polyadenylation: One gene, many proteins

Processing of pre-mRNAs to translation-competent mRNAs is a complex and tightly regulated multi-step process that results in the accurate removal of intronic sequences by splicing and the addition of a 5' cap and a 3' polyadenylation sequence [15].

The choice and usage of splice sites in the pre-mRNA can be differentially selected by AS, depending on the developmental state, tissue and cell type or in response to a variety of physiological stimuli or pathological conditions [16]. AS occurs in more than 90% of multi-exonic pre-mRNA transcripts and provides much of the proteomic diversity observed in mammalian cells [17]. AS can also downregulate expression through the introduction of premature termination codons (PTCs) by inducing frame shifts in the open reading frame. PTCs trigger the nonsense mediated decay (NMD) pathway, thus marking a transcript for degradation [18].

Polyadenylation is a co-transcriptional nuclear process essential for efficient nuclear export, stability and translation of mRNAs [19,20]. The process involves the endonucleolytic cleavage of the pre-mRNA, followed by the addition of a polyA tail. The specific cleavage location is controlled by cis-acting motifs, including a hexamer polyadenylation signal (PAS) and a downstream UG-rich element that aids in the recruitment of the polyadenylation machinery [20].

Like AS, APA is prevalent and affects over 50% of human genes. APA is commonly assumed to occur 'in tandem' within a given UTR, but recent analysis has shown that alternative PAS are also frequently present in 'upstream' intronic regions [21,22]. While tandem APA contributes mainly to the regulation of the *levels* of expression of one encoded product, intronic polyadenylation (IPA) also adds to the *diversity* of protein isoforms generated from a single gene. For example, tandem APA motifs can affect gene expression levels or transcript stability by removing/adding regulatory sequences, such as micro-RNA binding sites and/or AU-rich elements (AREs) [23]. On the other hand, the usage of IPA motifs will generate truncated mRNAs that could produce protein isoforms with potentially vastly different functions than their full-length counterparts, including dominant-negative characteristics.

Collectively AS and IPA are key molecular mechanisms for increasing the functional diversity of the human proteome, allowing the relatively small human genome (<25,000 genes) to generate an excess of 100,000 different protein isoforms [17].

Alternative Splicing and Polyadenylation in Cancer

The alternative splicing pattern of individual pre-mRNAs is a complex process primarily controlled by the intrinsic strength of the splice sites, by the combinational effects of competitive cis-regulatory elements within the pre-mRNA (intronic or exonic enhancers or silencers) and by the relative abundance of the trans-acting factors that recognize them (such as SR and hnRNP proteins) [24,25].

Given AS complexity and its pivotal role in the control of gene expression and functions, it is not surprising that many human genetic diseases are a direct consequence of aberrant splicing events. Indeed,

approximately 50% of disease-associated point mutations directly affect splicing [15,26]. These typically result from mutations that impair specific splice sites or other regulatory *cis*-acting elements, or that activate cryptic sites, as it can occur, for example, in β-Thalassaemia [27].

A growing body of evidence suggests that deregulated AS is also extensively linked to the development of cancer. Cancer cells show aberrant splicing profiles, expressing isoforms that can stimulate cell proliferation, enhance survival, promote migration and invasion, influence metabolism and in general affect any aspect of tumor progression and maintenance [28]. As in genetic diseases, this can sometimes be gained through acquisition of mutations in *cis*-acting AS elements, like for example with splice-site mutations identified in the BRAC1 and APC tumor suppressor transcripts [29,30]. More commonly however, the pathological splicing patterns in tumors are due to broader changes in the expression, activity and possibly specificity of regulatory trans-acting RNA binding proteins which might in part reflect regulated AS programs that are improperly activated [28,31] or to mutations in splicing factor or core spliceosomal components [32].

Indeed, many genes that play a key role in tumor development, invasiveness or chemo-resistance express multiple splicing isoforms, with very different and often antagonistic functions. The idea of a more direct role of splicing in tumorigenesis is also supported by the observation of oncogene-like behavior for some splicing factors, which can act as very early markers of cancer (e.g.: hnRNP A2/B21 in lung tumors [33]), can carry frequent activating mutations (e.g.: SRSF2, SF3b1 and others in Myelodysplasia [32,34]) or, like SRSF1, can directly behave as powerful single-agent oncogenes in mouse models and are found to be amplified in some tumors [35,36]. Similarly, the splicing factor hnRNP-H is up-regulated and contributes to the pathogenesis and progression of glioblastoma multiforme (GBM) by inducing oncogenic alternative splicing isoforms of Receptor d'origine nantais (RON) and Insuloma-glucagonoma protein 20 (IG20) which promote migration and survival, respectively, a switch that might reflect the re-activation of a stem-like gene expression program in the GBM cells [37].

Although not as well characterized as AS, recent genome-wide analysis of APA also points to its de-regulation as being linked to tumorigenesis by increasing cellular proliferation and transformation. Cancer cells preferentially express oncogenes with shorter 3'UTRs, resulting in higher expression due to the loss of destabilizing and/or repressive elements from the UTR [38,39]. Alternatively, the modulation of upstream IPA sites can generate truncated variants with tumorigenic capabilities. For example, the usage of an IPA site in Cyclin D1, which normally controls progression through the cell cycle, results in a constitutively active truncated Cyclin D1b isoform, that can lead to cellular transformation [40,41].

Therefore, deregulation of AS and APA/IPA can contribute to tumorigenesis due to inactivation of tumor suppressors, activation of oncogenes and generation of novel pathogenic cancer-specific isoforms.

Splice-switching oligonucleotides

Since aberrant AS and APA contribute to a multitude of different human diseases, correction of such defective pre-mRNA processing by ASOs could open an avenue for therapeutic intervention. The feasibility of this approach has been elegantly demonstrated in the development of ASO compounds for the treatment of β-Thalassaemia [42] Spinal Muscular Atrophy (SMA) [43,44], and Duchenne Muscular Dystrophy (DMD) [45]. Systemic delivery of splice-switching oligonucleotides (SSOs) has proven to be highly effective in pre-clinical mouse models of these genetic disorders, where a shift in endogenous splicing has been induced to correct an aberrant splicing pattern or induce expression of therapeutic splice variants, with significant physiological responses [42-44]. Furthermore, exciting early results from clinical trials of compounds directed at the treatment of DMD have shown benefit in patients, indicating that the technology is finally maturing for clinical applications [46,47].

The principle of inducing therapeutic variants whilst reducing deleterious ones can also be applied as a cancer therapy approach, especially since many key 'cancer genes' can generate antagonistic isoforms, which often have dominant-negative characteristics. In other words, the dual role of these proteins can be exploited by splicing re-direction approaches to manipulate their expression, in order to simultaneously eliminate the deleterious isoforms and substitute them with therapeutically desirable ones. This would be particularly effective when the targets are essential to survival of cancer, as in the case of "oncogene addiction", where cancers remain dependent upon a primary oncogenic driver activity for their continued survival and malignant progression [48].

Here we describe some examples using this rationale to modulate AS and IPA and redirect splicing to generate antagonist or therapeutic mRNA variants that function as potential novel cancer therapies.

The anti-Warburg effect: Switching off PK-M2

Cancer cells exhibit altered metabolic pathways, to derive energy to sustain their aberrant physiology. Normally, differentiated and non-proliferating cells generate the bulk of their energy requirements from mitochondrial oxidative phosphorylation. However, in cancer cells, energy is mainly generated through aerobic glycolysis, converting glucose to lactate, in a phenomenon known as *the Warburg effect* [49].

To achieve this metabolic switch, cancer cells reprogram their energy metabolism pathways by

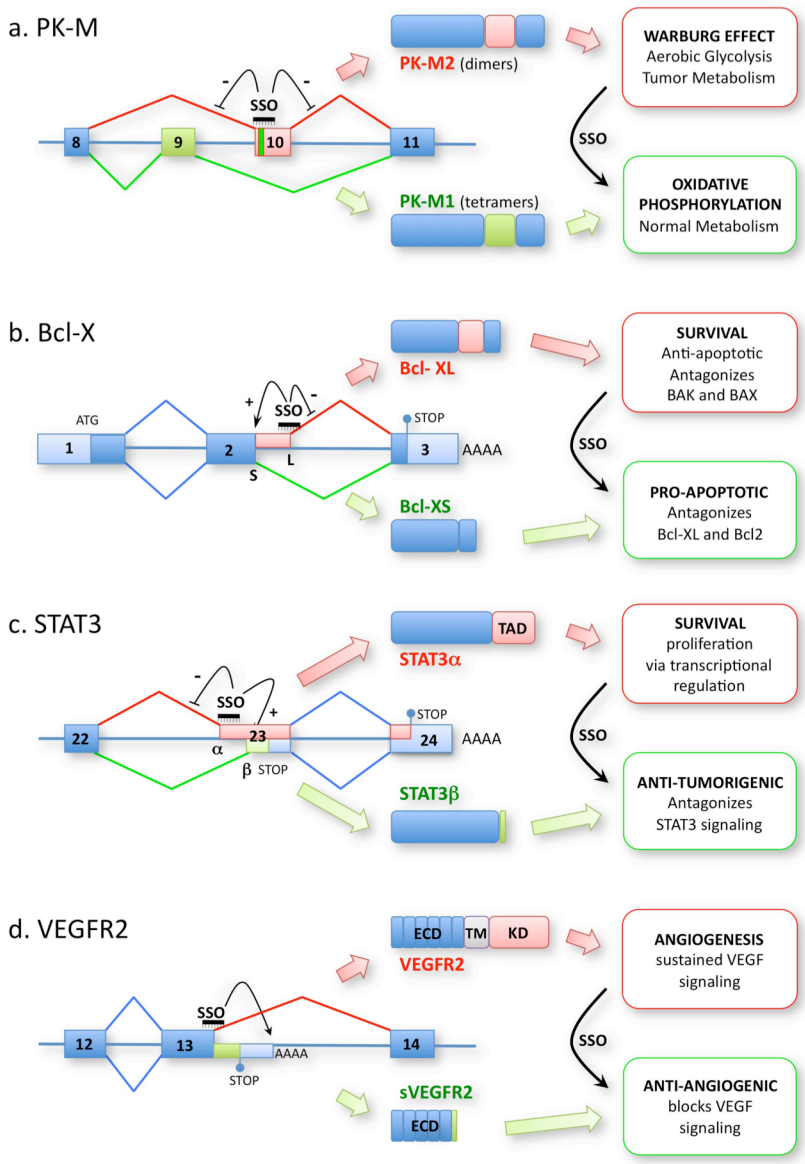


Figure 1. Examples of Therapeutic Potential of Modulating AS and APA Events in Cancer. *SSO can be employed to redirect pre-mRNA processing to induce therapeutic anti-tumorigenic isoforms. (a) SSO targeting an ESE in exon 10 of PK-M produces an isoform (PK-M1) which antagonizes the Warburg effect. (b) SSOs targeting the alternative down-stream 5' ss in exon 2 of the Bcl-X pre-mRNA, modulates AS to produce BcL-XS, a pro-apoptotic isoform. (c) SSO targeting a ESE in exon 23 of STAT3 shifts AS to produce STAT3β, an anti-tumorigenic isoform. (d) SSO targeting the 5' SS of exon 13 in VEGFR/KDR activates IPA, producing a soluble VEGFR/KDR isoform with anti-angiogenic and anti-mitogenic properties.*

multiple mechanisms, including by changing the splicing pattern of pyruvate kinase M (PK-M). PK-M functions as the rate limiting final step in the glycolysis pathway, converting phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP [50]. AS of the PK-M gene generates two isoforms, PK-M1 and PK-M2, through the mutually exclusive usage of Exon 9 and Exon 10, respectively [51]. PK-M1 is found principally in differentiated cells, while PK-M2 is expressed in normal proliferating tissues (e.g. during embryonic development), but it is also expressed in tumor cells [52]. The regulatory role of PK-M2 in metabolism is complex, but its general role is to alter cellular glucose metabolism, increasing the availability of glycolytic metabolites that are then utilized to support proliferation and growth. In tumors, these features are essential for cancer survival and progression [52].

The splicing regulation of the PK-M1/M2 switch involves both exonic splicing silencers (ESSs) in exon 9 and exonic splicing enhancers (ESEs) in exon 10, in conjunction with trans-acting RNA binding proteins SRSF3, PTB/nPTB, and hnRNPA1/A2, partially controlled by oncoprotein MYC [53-55]. In combination, they repress exon 9 inclusion and promote expression of the PK-M2 splice variant. 2'-MOE ASOs directed against an identified exonic splicing enhancer in exon 10 of the PK-M2 pre-mRNA were shown to inhibit exon 10 usage and re-activate PK-M1 expression by promoting exon 9 inclusion (Figure 1a). This resulted in downregulation of PK-M2 protein expression, with a concurrent increase of the PK-M1 isoform in a panel of glioblastoma cell lines. The switch was associated with reduced viability and increased apoptosis, thus validating this approach [55]. Although further *in vivo* work is required, the PK-M2 to PK-M1 switchback provides a therapeutic strategy to specifically target cancer cells based on their unique metabolic signature.

From survival to apoptosis: Switching between Bcl-XL and Bcl-XS

The ability of cancer cells to escape apoptotic programmed cell death is also a key aspect of the oncogenic process and of the appearance of resistance to drugs in tumors [2]. Central to the activation of the apoptotic program are the Bcl2 family of survival proteins, which includes Bcl-X [56]. Alternative 5'ss splicing in Bcl-X exon 2 generates two isoforms with opposite functions: Bcl-XL, a well-characterized survival protein and the smaller Bcl-XS, a pro-apoptotic variant [57].

Bcl-XL is up-regulated in a broad range of cancers and its over-expression is strongly associated with resistance to chemotherapy and poor clinical outcome. While Bcl-XL inhibits apoptosis through neutralizing hetero-dimerization with pro-apoptotic proteins Bak and Bax [58], its alternative isoform, Bcl-

XS induces apoptosis by directly inhibiting Bcl-XL and Bcl-2, and its levels are low in cancer cells despite the high expression of the Bcl-X gene [59]. The Bcl-XL/XS splicing regulation has been extensively investigated, and multiple controlling *cis*-elements and their cognate *trans*-acting splicing factors have been identified [60,61]. The long recognized antagonistic functions of Bcl-XL and Bcl-XS and their central role in controlling survival of cancer cells have made them a prime target of splicing reprogramming approaches.

Several studies have employed various ASO strategies to modulate the endogenous Bcl-X AS, to effectively activate the upstream Bcl-XS 5'ss at the expense of the downstream Bcl-XL one in exon (Figure 1b). This switch in Bcl-X pre-mRNA processing induces apoptosis and increased *in vitro* chemosensitivity in lung, prostate and breast cancer cells [62-64]. Furthermore, *s*ystemic delivery of such ASOs demonstrates that switching of Bcl-X pre-mRNA can be obtained *in vivo,* where it reduces tumor load and metastases in a mouse model of metastatic melanoma [64].

STAT3: the alpha to beta anti-tumorigenic switch

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of transcription factors, which are key down-stream effectors of gene expression following cytokine, hormone or growth factor stimulation [65]. STAT3 is persistently activated in a broad range of tumors, where it promotes growth and survival of tumor cells, induces tumor angiogenesis and suppresses anti-tumor immune responses [66]. Abnormal cytokine or growth factor signaling through the Janus kinases (JAK) and receptor tyrosine kinases (RTKs) such as EGFR, led to its aberrant hyper-phosphorylation, and its enhanced activation is often associated with acquired therapeutic resistance to chemotherapy and radiation therapy. Furthermore, STAT3 promotes resistance to targeted EGFR therapies in non-small cell lung carcinoma (NSCLC) and head and neck squamous cell carcinoma (HNSCC) [67,68]. Because of this position at the center of many converging signaling and oncogenic pathways, STAT3 has become an attractive target for cancer therapy. Several strategies have been developed to target the STAT3 pathway *in vivo*, including small drug inhibitors, peptide analogs, decoy binding-site oligonucleotides and over-expression of STAT3β, an alternative isoform of STAT3 that acts as a dominant negative/antagonist variant of STAT3 [69,70].

The truncated STAT3β isoform is generated from usage of an alternative 3'ss of exon 23, 50 nucleotides downstream of the normal 3'ss [71]. The resulting stop codon causes STAT3β to lack 55 residues of the C-terminal transactivation domain (TAD). STAT3β can still be phosphorylated at the Tyr705 position, essential for dimerization and nuclear translocation, and thus it retains the ability to bind target nuclear DNA sequences as a homo-dimer or hetero-dimer (with full length STAT3 α, or other transcription factors). As a consequence, STAT3β can act as a dominant negative regulator as well as mediate additional distinctive features that add to the complex STAT3 signaling cascade [70,72,73].

Phosphorodiamidate morpholinos (PMOs) targeting an identified ESE in Exon 23 of STAT3 can quantitatively switch the endogenous mRNA from the alpha to the beta form in multiple cell types (Figure 1c) [73]. *In vitro*, antisense modulation of STAT3β/α ratios results in a reduction in cell viability and an increase in apoptosis, higher than those observed in a parallel total STAT3 knockdown experiment [73]. The enhanced biological effect of the alpha to beta switch compared to the knockdown appears to be due to the activation of a unique STAT3β-specific regulation program with anti-tumorigenic characteristics, rather than a direct block of canonical STAT3 targets. Conjugation of the STAT3β-inducing PMOs to a cell-penetrating dendrimer (vivo-morpholinos [74]) allows redirection of STAT3 splicing *in vivo* and concomitant tumor regression in a breast cancer xenograft model [73]. Remarkably, under comparable experimental conditions, effective knockdown of STAT3 has no significant effect on tumor growth, underscoring the qualitative difference in reprogramming versus knockdown approaches and the vast, untapped potential of splicing redirection in cancer therapy.

Activation of intronic polyadenylation (IPA): Induction of secreted decoy RTKs

Activation of the RTK signaling pathways represents a key aspect of tumorigenesis in a broad range of human cancers [75,76]. The targeting of oncogenic RTKs provides a classic example of molecular targeted therapeutic approach in modern cancer therapy. Effective targeted therapies to RTKs include small molecule tyrosine kinase inhibitors and monoclonal antibodies [77]. However, resistance invariably emerges over time, and secondary lines of treatment need to be developed for relapsing patients.

An alternative approach to target RTK receptor pathways has been the delivery of recombinant soluble, dominant-negative RTKs variants (secreted decoy RTKs, sdRTKs) comprising the sole ligand-binding, extracellular domain (ECD), by either direct intravenous administration or using gene-therapy viral expression vectors. SdRTKs antagonize RTK signaling by binding and sequestering the activating ligand and/or by engaging the endogenous receptor in non-productive dimers (Figure 2). An example of this approach is Aflibercept, a VEGF trap that suppresses tumor growth and vascularization *in vivo* and is currently in Phase III clinical trials [78].

A recent study demonstrated the existence of tens of natural endogenous sdRTKs, which are expressed from most RTK genes, where they are generated by the activation of IPA sites in introns upstream of the transmembrane domain-encoding

exons [79]. The controlled and specific induction of such sdRTKs can be obtained by ASO targeting the 5' splice site immediately upstream of an IPA signal [79]. Normally, directing an ASOs to the 5'ss would interfere with splicing, typically resulting in exon skipping. However, if a *bona fide* PAS is present just downstream in the intron, targeting the 5'ss activates the intronic PAS to generate a truncated mRNA by a U1snRNP-dependent mechanism (Figure 2).

U1snRNP plays an essential role in pre-mRNA splicing, where it is needed to select and define 5'ss by base-pairing with its own snRNA [80]. In addition, U1snRNP can also directly inhibit polyadenylation when tethered to the 3'UTR of a gene [81,82]. This suppression by U1 is not limited to the 'normal' 3' UTR, but is in fact a general non-splicing function of U1, possibly as part of the RNA surveillance machinery [79,83]. Since in any pre-mRNA, every intron 5'ss is bound by U1 during splicing, downstream IPA sites are silenced as a consequence. Hence, preventing U1 binding at the 5'ss would relieve suppression and allows IPA to occur, in a splicing-independent mode.

On the basis of these observations, ASOs were designed to specifically block U1snRNP binding to individual 5'ss sites upstream of the IPA sites corresponding to various natural sdRTKs (EGFR, MET, HER2, VEGFR1 and VEGFR2), resulting in their activation in place of the endogenous full length RTKs [79]. This was exemplified by targeting of VEGFR2, the main mediator of VEGF signaling. Dysregulation of signaling via this receptor is common in tumorigenesis, with mitogenic and angiogenic consequences. Vivo-morpholino ASOs were used to induce endogenous sVEGFR2 by IPA activation [79] (Figure 1d) and to suppress VEGF signaling and angiogenesis in a paracrine and autocrine fashion *in vitro* and *in vivo* [79,84], reproducing the inhibitory activity of the natural sVEGFR2 variant [85].

A similar approach has been applied to HER2/ErB2, a member of the EGFR family of receptor kinases, which is over-expressed in many human malignancies and is prevalent in breast cancer [86]. To activate endogenous soluble HER2 receptors, a 2'-MOE ASO was directed against the 5'ss of exon 15 of HER2, inducing exon skipping and leading to a frameshift and the putative use of a premature stop codon upstream of the transmembrane domain [87]. The ASO treatment was associated with an increase in apoptosis and inhibition of proliferation in breast cancer cell lines. Interestingly, intron 15 of HER2 also contains an intronic PAS, which can be activated by targeted PMOs [79], suggesting that the observed truncated version and associated biology may be generated by IPA activation rather than (or in addition to) NMD-escaping exon-skipping.

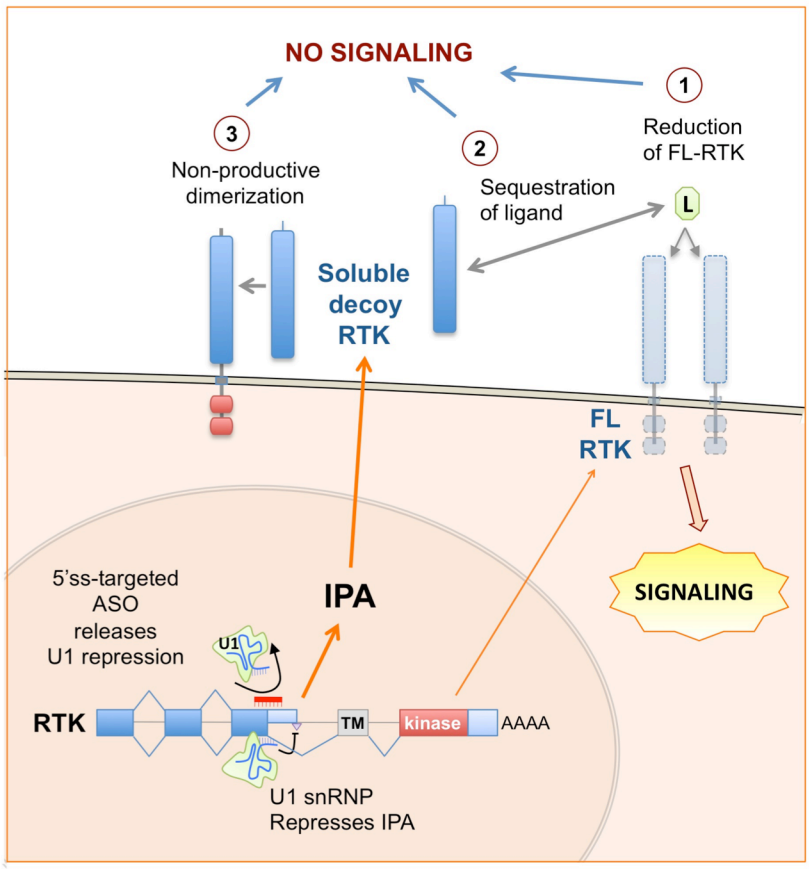


Figure 2. Effects of IPA activation on RTK signaling. *Splicing re-direction compounds block U1 snRNP suppression of an intronic PAS upstream of the exon encoding the transmembrane (TM) domain, and activate IPA. This inhibits signaling by three simultaneous mechanisms: 1) direct reduction of the levels of active FL-RTK; 2) sequestration of ligand; 3) inhibition of residual FL-RTK or other compatible RTKs by engaging them in non functional hetero-dimers. Adapted from Vorlova et al, Mol Cell, 2011.*

In general, the generation of sdRTKs is a potent approach to target oncogenic signaling pathways via three potential scenarios (Figure 2) that occur simultaneously upon addition of a single ASO compound: 1) concomitant downregulation of full-length oncogenic RTK; 2) secretion of a ligand-binding soluble receptor leading to its sequestration; 3) non-productive dimerization with residual receptors or co-receptors.

The result is a powerful approach that recapitulates, by use of a single compound, the application of multiple inhibitory strategies: knockdown of the gene's expression, trapping of the ligand and trans-inactivation of the receptor, all of which are widely used to block signaling RTK activities in different experimental systems and therapeutic scenarios.

Overall, given the essential and central role of aberrant RTK signaling in a broad range of cancers and the existence of sdRTK variants for most of them, ASO-mediated activation of IPA and the induction of endogenous antagonistic RTK isoforms holds a tremendous therapeutic potential in cancer therapy.

Conclusion

The application of large scale cancer genome sequencing and molecular profiling has opened the door to tackling cancer through the identification of genes and networks essential to the survival of a tumor and/or its malignant progression. It has also permitted the development of highly specific molecular targeted therapies, traditionally based on small molecule compounds or monoclonal antibodies. Advances in nucleic acid chemistry and pharmacology indicate that antisense-based therapies may finally fulfill their great potential as a *bona-fide* alternative drug discovery platform to develop novel anti-cancer agents.

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